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13. ABSTRACT (Maximum 200 Words)

The BRCA2 tumor suppressor gene has been suggested to be important for DNA repair and maintaining genome integrity. Most evidences supporting this hypothesis, however, were obtained from studying mouse embryonic cells. The importance of BRCA2 in maintaining genome integrity in human cells is not very clear. We have completed all tasks of this study. We have generated derivatives of Capan-1 cells, which expressed only mutant BRCA2, that express wild-type BRCA2. Characterization of Capan-1 derivatives showed that expression of wild-type BRCA2 did not have a detectable effect o the sensitivity to γ-irradiation or DNA damaging drugs of Capan-1 cells. Characterization of MCF12A derivatives showed that reduction of BRCA2 diminished the formation of γ-irradiation induced RAD51 nuclear foci and increased the sensitivity to γ-irradiation of MCF12A cells. Unexpectedly, we also found that expression of wild-type BRCA2 suppressed the growth of Capan-1 cells in vitro and in vivo. On the other hand, reduction of BRCA2 appears to inhibit the growth of MCF7 cells.

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INTRODUCTION

Germline mutations of the tumor suppressor gene *BRCA2* have been shown to predispose

their carriers to breast and ovarian cancers (1, 2) and to increase their risk for many other types

of cancer (3-5). As is the case with many other tumor suppressor genes, the wild-type allele of

the *BRCA2* locus is frequently lost in tumors of patients carrying a mutant *BRCA2* allele (3, 6-8).

However, unlike those of most tumor suppressor genes but similar to those of *BRCA1*, somatic

mutations of *BRCA2*, are rarely found in sporadic breast or ovarian cancers (9-13). Furthermore,

mutation analysis of *BRCA2* in more than 200 cell lines derived from a variety of human cancers

has identified only one pancreatic cancer cell line (Capan-1) that carries a hemizygous mutation

(12). The *BRCA2* mutation found in Capan-1 is 6174delT (12, 14), a mutation found frequently

in Ashkenazi Jews and one that clearly predisposes its carriers to a variety of cancers (3, 15-19).

These observations strongly indicate that the Capan-1 cell line was derived from a patient with

germline *BRCA2* mutation.

The *BRCA2* gene encodes a transcript that is larger than 10 kb and is predicted to produce

a protein of 3418 amino acids (2). The BRCA2 protein preferentially localizes to the nucleus (20,

21). Analysis of the predicted BRCA2 amino acid sequence has identified several internal

repeats (22, 23). These BRC repeats are highly conserved among different species but lack

homology with any known functional domain. The BRCA2 protein has been shown to interact

with RAD51 through its BRC repeats and carboxyl terminus (21, 24-28).

The *Saccharomyces cerevisiae* RAD51 belongs to the RAD52 epistasis group that is

essential for repairing double-strand DNA breaks (DSB) by homologous recombination

(reviewed in (29). The *Rad51* gene is essential for mouse embryonic development in vivo and for

the growth of the chicken DT40 cell line in vitro (30-32). Mouse embryo cells without *Rad51* are

hypersensitive to ionizing radiation and have reduced DNA content, while DT40 cells without *Rad51* exhibit a high frequency of chromosomal breakage before death (30, 32). Inactivation of *Rad54*, a member of the *Rad52* group, also increases sensitivity to ionizing radiation (33, 34). In addition, *Rad52* and *Rad54* are important for homologous recombination in vertebrate cells (33-36). Mouse cells lacking a functional *Brca2* gene also have been shown to be deficient in repairing DNA damage (24, 37-40).

The mechanism for the tumor suppression function of *BRCA2* in human is not understood. However, the identification of the association of BRCA2 with RAD51 and studies of *Brca2* knockout mice have led to the suggestion that BRCA2 participates in RAD51-mediated repair of DSB (24, 37, 38, 41, 42). A clearer understanding of the functions of BRCA2 will lead to a better understanding of tumorigenesis in mutant *BRCA2* carriers. It also could have a great impact on the understanding of breast cancer development in general. One major difficulty in studying the function of *BRCA2* is that there is no human breast cancer cell line that does not express wild-type BRCA2. The only human cell line that does not express wild-type *BRCA2* is Capan-1, a pancreatic cancer cell line. Although Capan-1 has been shown to be hypersensitive to DNA-damaging agents (21, 43), it is not clear whether this property is common to other human cells lacking functional *BRCA2*.

This study was designed to determine whether BRCA2 has a role in DNA repair in human somatic cells. The objectives of this study are:(1) Generation of derivatives of the pancreatic cancer cell line Capan-1 that conditionally express wild-type BRCA2, (2) Generation of derivatives of the normal breast epithelial cell line MCF-12A and the breast cancer cell line MCF7 that do not express wild-type BRCA2, and (3) Characterization of the effects of altered

expression of BRCA2 on the genomic integrity of cells. In the past three years of this study we have accomplished all these tasks.

BODY

Materials and Methods

Cell lines. Capan-1 is a human pancreatic cancer cell line that expresses only truncated BRCA2. HCT116 is a colon cancer cell line, MCF12A is a normal human breast epithelial cell line, MCF7 is a breast cancer cell line and Du145 is a prostate cancer cell line.

Plasmids. We isolated the cDNA for the entire coding region of *BRCA2* by reverse transcription (RT)-polymerase chain reaction (PCR). Because of its large size, the *BRCA2* coding region was divided into four fragments for RT-PCR. Several clones of each amplified fragment were sequenced to identify those did not contain any mutation resulted from the PCR reaction. These fragments were sequentially ligated together to obtain the full-length cDNA for *BRCA2*. The *Xho*I restriction site was engineered at the both ends of the assembled *BRCA2* cDNA. To facilitate the assembly of the full length coding cDNA of *BRCA2*, codon 798 was changed from CTC to CTT to create a *Hind*III restriction site; however, this change does not alter the encoded amino acid. The *BRCA2* cDNA was inserted into a derivative of pUHD10-3 (44) to construct a plasmid that expresses BRCA2 under the control of a tetracycline-regulated promoter.

The plasmid expressing small interference RNA (siRNA) targeting *BRCA2* was generated according to a published strategy (45). Plasmids expressing two siRNA targeting the sequences 5'-GAGCAGCATCTTGAATCTC-3' and 5'-GGAGGACTCCTTATGTCCA-3' of *BRCA2* were first generated and then the two transcription units were combined in a single plasmid in a head-to-tail direction.

Generation of Capan-1 derivatives. The control clones, Control 1 and 2, and tetracycline-regulated wild-type BRCA2 expressing clones, Clones A and B, were generated similar to described above but used a plasmid expressing tetracycline regulator (tTA), tTA-IRES-Neo (46), together with a derivative of pUHD10-3 (44) that expressed *BRCA2* at 1:9 ratio using Lipofectamine Plus (Life Technologies) then selected with G418. The two control clones express functional tTA as determined by tTA-reporter assay, but do not express wild-type BRCA2 as determined by immunoblotting.

Clones C1 and C2 are Capan-1 derivatives that constitutively express the wild-type BRCA2 and were generated similar to those described above but used a plasmid expressing the reverse tetracycline regulator (rtTA) instead of one expressing tTA. Two wild-type BRCA2 expressing clones were obtained but the expression of the wild-type BRCA2 was constitutive instead of regulated.

Generation of MCF12A derivatives. MCF12A cells were transfected with pCMV-NEO-BAM (47) together with a plasmid expressing two siRNA targeting *BRCA2* at 1:9 ratio using Lipofectamine Plus (Life Technologies) then selected with G418. Levels of BRCA2 expressed in selected clones were determined by using immunoblotting.

Immunoblotting, immunoprecipitation and immunofluorescence staining. Detection of BRCA2 by immunoblotting was performed as previously described by using the BRCA2 antibodies N61 (48). RAD51 was detected by using a monoclonal antibody purchased from GeneTex (San Antonio, TX).

For immunoprecipitation, cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0 and 0.5% Nonidet P-40. Immunoprecipitation of BRCA2 was carried out using

a rabbit polyclonal antibody against the C terminus of BRCA2 (Ab-2, Oncogene Science, Cambridge, MA). BRCA2 proteins were detected by immunoblot analysis as described above.

RAD51 in cells was detected by immunofluorescence staining. Cells were plated on cover glass and were irradiated with 10 Gy of γ-irradiation two days later.

Determination of sensitivity to γ-radiation by clonogenic assay. Parental Capan-1 cells and Capan-1 derivatives constitutively expressing wild-type BRCA2 were plated on 60-mm cell culture dish at the density of 1,000 cells per dish. Cells were irradiated with various doses of γ-radiation two days after plating and were cultured for another 16 days with media changed every two days. Survived colonies were stained with crystal violet.

Capan-1 derivatives expressing wild-type BRCA2 regulated by tetracycline were plated on each 60-mm dish at the density of 1,000 cells per dish in the presence of tetracycline. Each cell line was plated on 20 dishes. Tetracycline was removed from 10 dishes of each cell line two days later to induce the expression of wild-type BRCA2 (Induced). The remaining 10 dishes were maintained in tetracycline-containing medium (Uninduced). Cells were irradiated with various doses of γ-radiation another two days later. All cells were fed with tetracycline-containing media two days after the irradiation and cultured for another 11 (for Clone B) or 12 days (for Clone A) with media changed every two days. The survived colonies were stained with crystal violet.

Determination of sensitivity to γ-radiation by cell growth assay. Each clone of Capan-1 derivatives expressing wild-type BRCA2 regulated by tetracycline were plated on ten 60-mm dishes at the density of 50,000 cells per dish in the presence of tetracycline. Tetracycline was removed from 5 dishes of each cell line two days after plating to induce the expression of wild-type BRCA2, and these cells were maintained in tetracycline-free medium until the end of the

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experiment. Cells in the remaining 5 dishes were maintained in tetracycline-containing medium throughout the experiment. Cells were irradiated with various doses of γ-radiation 4 days after plating. The number of survived cells at 6 (for Clone B) or 7 (for Clone A) days after irradiation was determined by counting.

Parental MCF12A cells were plated on 60-mm cell culture dish at the density of 10,000 cells per dish and MCF12A derivatives expressing reduced levels of BRCA2 were plated on 60-mm cell culture dish at the density of 50,000 cells per dish. Cells were irradiated with various doses of γ-radiation two days after plating. The number of survived cells at 6 days after irradiation was determined by counting.

Determination of cell growth by clonogenic assay. Cells were plated in 6-well plate at a density of 1000 cells/well in the tetracycline-containing media. Cells were allowed to grow for 2-1/2 weeks with media changed every two days in the tetracycline-containing media all the time or in the tetracycline-free media beginning two days after plating.

Determination of tumor growth. Capan-1 cells and Capan-1 derivative cells were harvested by trypsinization, washed with PBS then suspend in PBS at the density of 1×10^7 cells/ml. Ten female nude mice of 6-8 weeks of age were each subcutaneously inoculated with 1×10^6 of cells on each side of their flanks. Five mice in each group had been fed with doxycycline (0.2 mg/ml) in drinking water for 5 days before the inoculation. The mice were continuously fed with water containing doxycycline or without doxycycline. The tumor volume was measured on the indicated days. Tumor volumes were determined by external measurement in two dimensions and calculated using the equation $V = [L \times W^2] \times 0.5$, where V is volume, L is length, and W is width.

Results

Generation of wild-type BRCA2-expressing Capan-1 derivatives

We first attempted to generate Capan-1 derivatives that expressed wild-type BRCA2 regulated by tetracycline. We transfected Capan-1 cells with a vector expressing tetracycline regulated activator, tTA-IRES-Neo (46), together with a vector expressing BRCA2 cDNA under the control of tetracycline regulated promoter. After screening about 140 clones obtained from two separate transfection experiments, we isolated two clones (clones A and B), one from each transfection, that expressed wild-type BRCA2 tightly regulated by tetracycline (Figure 1A). We also carried out transfection using a vector expressing reversed tetracycline regulated activator together with a vector expressing BRCA2 cDNA under the control of tetracycline regulated promoter. We identified two clones (clones C1 and C2) that express wild-type BRCA2, however, these two clones expressed wild-type BRCA2 constitutively (Figure 1B). Using co-immunoprecipitation, we demonstrated that the exogenous wild-type BRCA2 associated with RAD51 as expected (Figure 1C).

Sensitivity of Capan-1 derivatives expressing wild-type BRCA2 to DNA damages

We investigated whether expression of wild-type BRCA2 altered the sensitivity of Capan-1 cells to γ-radiation. We first compared the sensitivity to γ-radiation of parental Capan-1 cells and clones C1 and C2 that expressed wild-type BRCA2 constitutively by using a clonogenic assay. As shown in figure 2A, there is no detectable difference in the sensitivity to γ-radiation between Capan-1 and clones C1 or C2. We then compared the sensitivity to γ-radiation of clones A and B, which expressed wild-type BRCA2 regulated by tetracycline, between expressing and not expressing wild-type BRCA2. A clonogenic assay and an assay that counting survival cells were used. As shown in figures 2B and 3, there is no detectable difference in the

sensitivity to γ-radiation between clones A or B expressed or did not express wild-type BRCA2. Our results show that expression of wild-type BRCA2 do not change the sensitivity of Capan-1 cells to γ-radiation.

We also investigated whether expression of wild-type BRCA2 altered the sensitivity of Capan-1 cells to DNA damaging chemicals. We chose methyl methanesulfonate (MMS), mitomycin C, etoposide and mitoxantrone for this study. We performed this study three times and with duplicated experiments each time. Our results show that there is no apparent difference in the sensitivity to DNA damaging drugs between the Capan-1 cells that expressed and that did not express wild-type BRCA2 (Figure 4).

Generation of MCF12A derivatives expressing reduced levels of BRCA2

We generated MCF12A derivatives that expressed reduced levels of BRCA2 by transfecting MCF12A cells with a plasmid expressing two siRNAs targeting *BRCA2*, together with a plasmid expressing G418 resistant gene. As shown in figure 5, we have obtained two clones (clones 1 and 2) that express reduced levels of BRCA2.

Sensitivity of MCF12A derivatives expressing reduced levels of BRCA2 to DNA damages

It has been reported that BRCA1 is required for γ-radiation induced RAD51 nuclear foci formation (49). We therefore first examined whether there was difference in forming RAD51 nuclear foci between MCF12A cells and MCF12A derivatives that expressed reduced levels of BRCA2 upon γ-irradiation. As shown in figure 6, RAD51 nuclear foci were formed in the majority of MCF12A cells but were rarely detectable in clone 2 that expressed reduced levels of BRCA2. Clone 1 also formed very few γ-irradiation induced RAD51 nuclear foci (data not shown).

We next examined the viability of parental MCF12A cells and MCF12A derivatives that expressed reduced levels of BRCA2 after γ-irradiation by counting survived cells. As shown in figure 7, both clones 1 and 2 cells were more sensitive to γ-irradiation than did the parental MCF12A cells.

Suppression of Capan-1 cells growth by wild-type BRCA2

While we were investigating the sensitivity to γ-irradiation of Capan-1 cells and Capan-1 derivatives that expressed wild-type BRCA2 by clonogenic assay, we noticed that clones A grew much slower when it was induced to express wild-type BRCA2 than when it was not. We examined the suppression of Capan-1 cells growth by wild-type BRCA2 in detail. As shown in figure 8, expression of wild-type BRCA2 suppressed the growth of both clones A and B.

Expression of wild-type BRCA2 suppressed the growth of Capan-1 cells not only in vitro but also in vivo. As shown in figure 9, expression of wild-type BRCA2 suppressed the growth of tumors resulting from clone A.

These results have been published (50).

BRCA2 may be essential for the growth of breast cancer cells

Our attempts to obtain MCF7 derivatives expressing reduced levels of BRCA2 resulted in only three G418 resistant clones in two separated transfection experiments and none of these clones expressed reduced levels of BRCA2. We therefore performed more detailed investigation of this observation. As shown in figure 11, although colonies were formed in MCF7 cells transfected with a plasmid expressing a control siRNA, no colony was form in cells transfected with the same plasmid expressing siRNA targeting BRCA2 used to generate MCF12A derivatives expressing reduced levels of BRCA2.

Discussion

We show that reduction of BRCA2 levels impairs the formation of RAD51 nuclear foci in MCF12A cells treated with γ-irradiation and increase the sensitivity of MCF12A cells to γ-irradiation. These observations are consistent with suggestions that BRCA2 plays an important role in repairing double-stranded DNA breaks (24, 37-40). On the other hand, our results show that expression of wild-type BRCA2 does not have detectable effect on the sensitivity to DNA damage of Capan-1 cells. Our observations are in contrast to that of expressing human wild type BRCA2 reduces the sensitivity to γ-irradiation and mitomycin C in a Chinese hamster ovary (CHO) cell line that expresses only endogenous mutant Brca2 (51). It is unlikely that the BRCA2 expressed in our Capan-1 derivatives has undetected mutations, we sequenced the entire *BRCA2* cDNA used in our study. Moreover, we showed that the wild type BRCA2 associates with RAD51 in Capan-1 cells and suppressed the growth of Capan-1 cells. A more likely explanation for difference between our results of studying Capan-1 cells and those of studying CHO cells is that Capan-1 cells are derived from human pancreatic cancer and may have other genetic alterations that affect the DNA repair.

We show that BRCA2 has paradoxical effects on cells growth. On one hand expression of the wild-type BRCA2 appears to suppress the growth of Capan-1 cells, which express only endogenous mutant BRCA2. On the other hand, reduction of levels of wild-type BRCA2 appears to inhibit the growth of MCF7 cells. It would be very important to elucidate mechanisms for how BRCA2 regulate cell growth.

Figure legends

Figure 1. Expression of wild-type BRCA2 in Capan-1 derivatives.

(**A, B**) Tetracycline regulated and constitutive expression of wild-type BRCA2. Clones A and B expressed wild-type BRCA2 under the regulation of tetracycline whereas Clones C1 and C2 expressed wild-type BRCA2 constitutively. Clones A and B were not induced (U), or induced (I) to express wild-type BRCA2 for one (I-1) or two (I-2) days in panel (B). (**C**) Association of RAD51 with wild-type BRCA2. Clone A cells were not induced (U), or induced (I) to express wild-type BRCA2 for two days and cell lysates were prepared. BRCA2 and RAD51 proteins in the total cell lysate (Total) or in the complexes immunoprecipitated by using an anti-BRCA2 antibody (IP) were detected by immunoblotting. The solid and dashed arrows indicate the wild-type and truncated BRCA2, respectively.

Figure 2. Sensitivity of Capan-1 derivatives to γ-radiation determined by clonogenic assay.

(**A**) Sensitivity to γ-radiation of parental Capan-1 cells and Capan-1 derivatives expressing wild-type BRCA2. One thousand cells were plated on each 60-mm cell culture dish. Cells were irradiated with the indicated doses of γ-radiation two days after plating and were cultured for another 16 days with media changed every two days. Survived colonies were stained with crystal violet. The top panel illustrates the time line of the experiment and the lower panel shows the result of a triplicate experiment. (**B**) Sensitivity to γ-radiation of Capan-1 derivatives expressing wild-type BRCA2 regulated by tetracycline. One thousand cells were plated on each 60-mm dish in the presence of tetracycline. Each cell line was plated on 20 dishes. Tetracycline was removed from 10 dishes of each cell line two days later to induce the expression of wild-type BRCA2

(Induced). The remaining 10 dishes were maintained in tetracycline-containing medium (Uninduced). Cells were irradiated with indicated doses of γ-radiation another two days later. All cells were fed with tetracycline-containing media two days after the irradiation and cultured for another 11 or 12 days as indicated with media changed every two days. The survived colonies were stained with crystal violet. The top panel illustrates the time line of the experiment. The lower panel shows the result of a duplicate experiment of Clones A and B as indicated.

Figure 3. Sensitivity of Capan-1 derivatives to γ–radiation determined by cell growth assay.
The top panel illustrates the time line of the experiment. The middle and the low panel show the result of Clones A and B, respectively. Each panel shows the results of three experiments. Fifty thousand cells were plated on each of ten 60-mm dishes in the presence of tetracycline. Tetracycline was removed from 5 dishes of each cell line two days after plating to induce the expression of wild-type BRCA2 (I), and these cells were maintained in tetracycline-free medium until the end of the experiment. Cells in the remaining 5 dishes were maintained in tetracycline-containing medium throughout the experiment (U). Cells were irradiated with indicated doses of γ-radiation 4 days after plating. The number of survived cells at 6 or 7 days after irradiation as indicated were determined by counting.

Figure 4. Sensitivity of Capan-1 derivatives to DNA damaging drugs.
One thousand indicated cells were plated on each well of 96-well plate in the presence of tetracycline. Tetracycline was removed from (I) or maintained in (U) the media to regulate the expression of wild-type BRCA2 two days after cell plating. Another two days later, cells were treated with indicated drugs at indicated doses. Cells were treated with MMS for one hour,

washed then cultured in tetracycline-free (I) or tetracycline-containing (U) media for two days. For all other drugs, cells were treated for two days in tetracycline-free (I) or tetracycline-containing (U) media. Survived cells were measured by using MTT assay. The results of three experiments are shown.

Figure 5. Reduction of BRCA2 expression in MCF12A derivatives.

BRCA2 and β-catenin in cell lysate containing equal amounts of total protein prepared from MCF12A, clone 1 and clone 2 as indicated were detected by immunoblotting.

Figure 6. Forming γ-irradiation induced RAD51 nuclear foci in a MCF12A derivative expressing reduced levels of BRCA2. MCF12A and Clone 2, which express reduced levels of BRCA2, cells were stained for RAD51 8 hr after treated with 10 Gy γ-irradiation. Cells were counterstained with DAPI to reveal nuclei.

Figure 7. Sensitivity of MCF12A derivatives to γ–radiation by cell growth assay.

The top and the bottom panels show results of two separate experiments.

Figure 8. Inhibition of the growth of Capan-1 cells by wild-type BRCA2 in vitro.

One thousand cells from each of the indicated Capan-1 derivatives were plated in each well of six-well plates in medium containing tetracycline. Cells were grown for 2-1/2 weeks, either in media containing tetracycline (Uninduced) or in tetracycline-free media (Induced) beginning 2 days after plating. The resulting colonies were stained with crystal violet. The results shown are from a triplicate experiment.

Figure 9. Inhibition of the growth of Capan-1 cells by wild-type BRCA2 in vivo.

Ten nude mice were subcutaneously inoculated with indicated cells on each side of their flanks.

Five mice in each group had been fed with doxycycline in drinking water for 5 days before the

inoculation. The mice were continuously fed with water containing doxycycline (uninduced,

open squares) or without doxycycline (induced, closed squares). The tumor volume was

measured on the indicated days.

Figure 10. Inhibition of the growth of MCF7 cells by siRNA targeting BRCA2.

MCCF7 cells were transfected with a plasmid expressing G418 resistant gene together with a

plasmid expressing indicated siRNA at a ratio of 1:9. Transfected cells were cultured in media

containing G418 for 30-35 days and the resulting colonies were stained with crystal violet. (A)

The result of experiment 1. (B) Summary of results of 4 independent experiments.
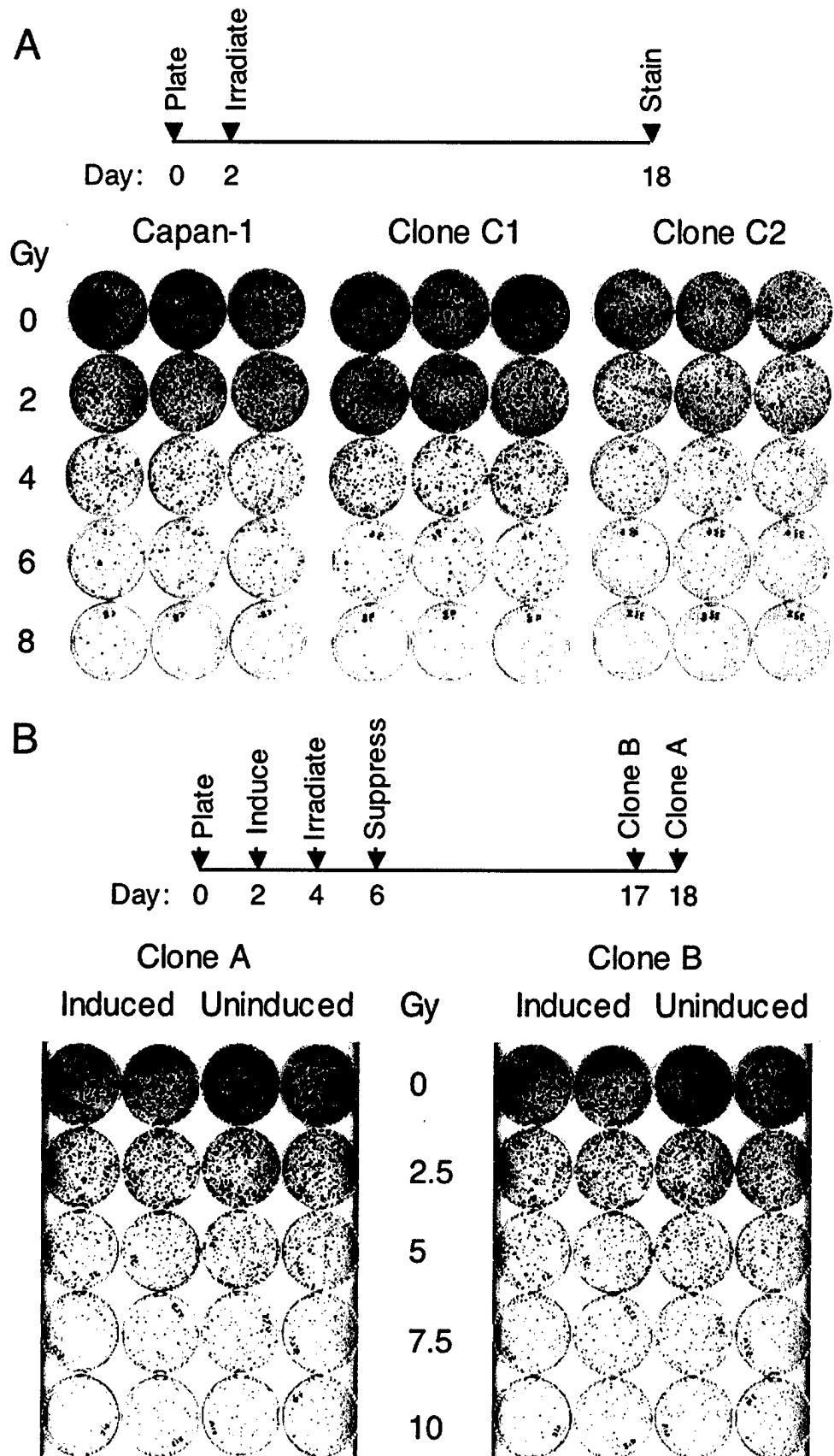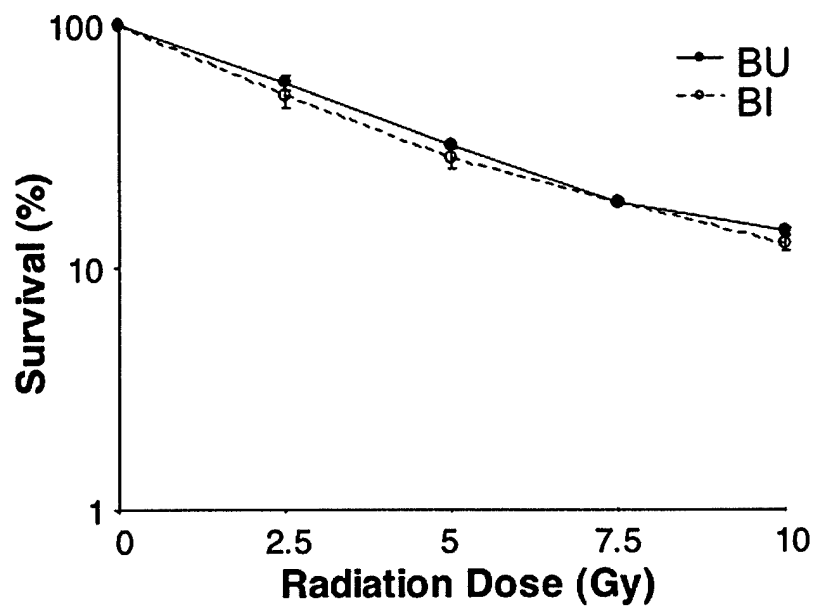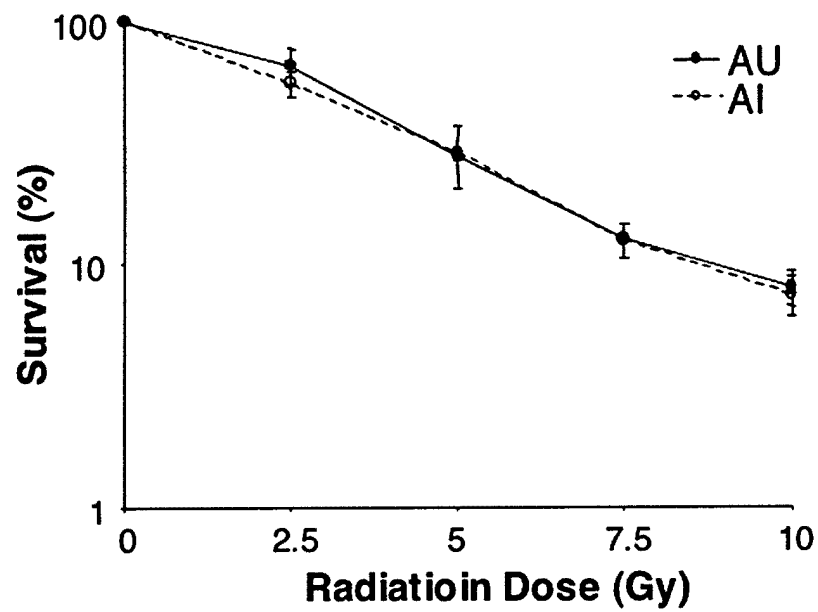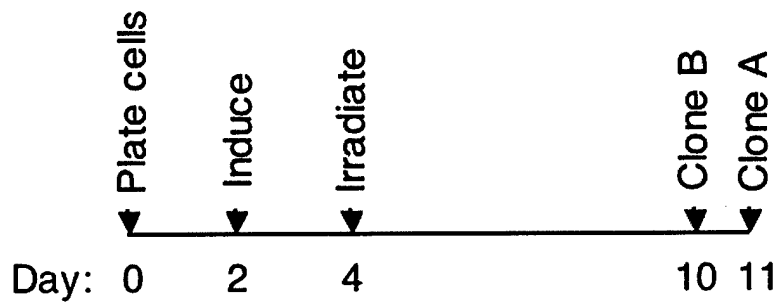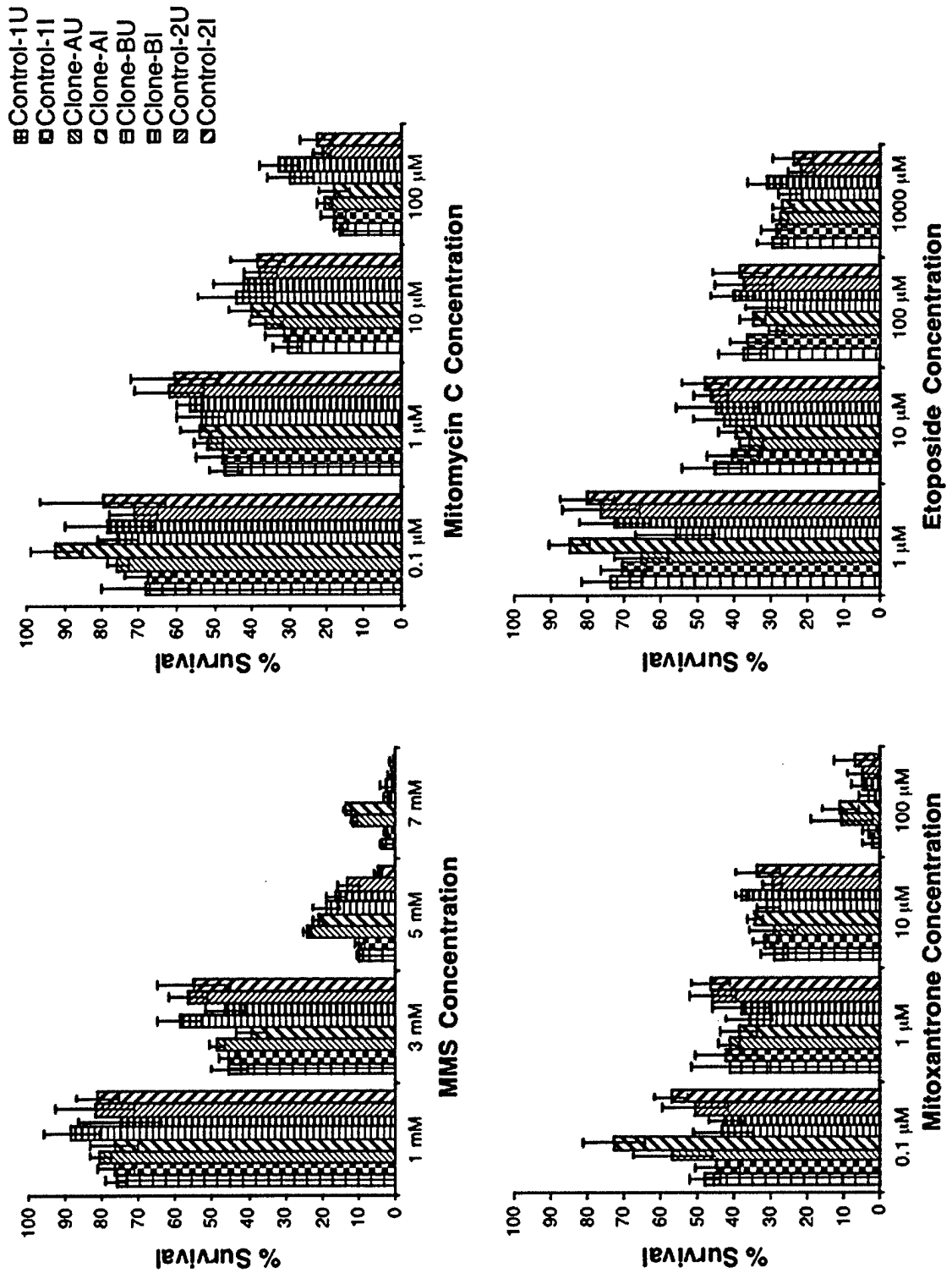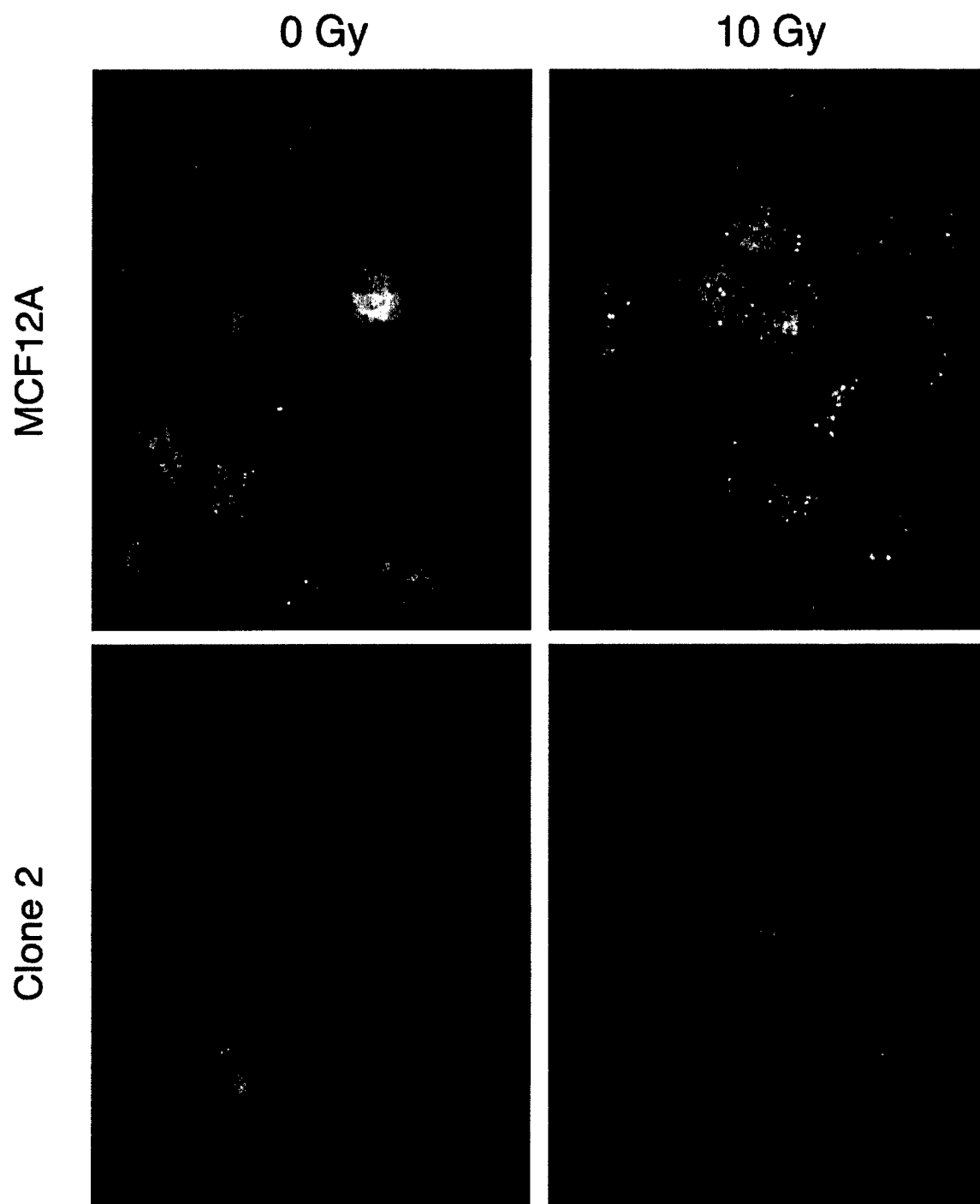
Figure 1

A

B

C
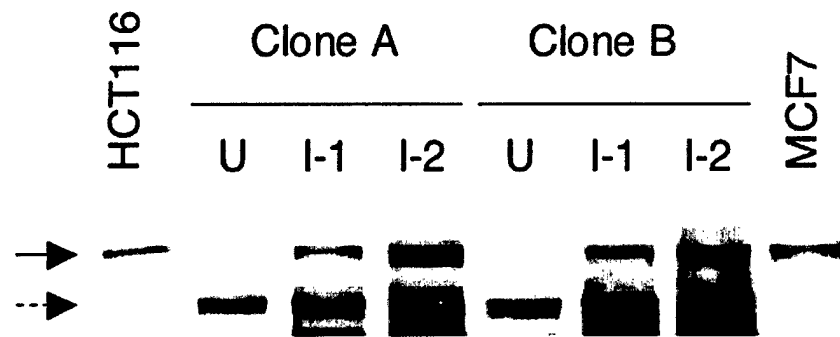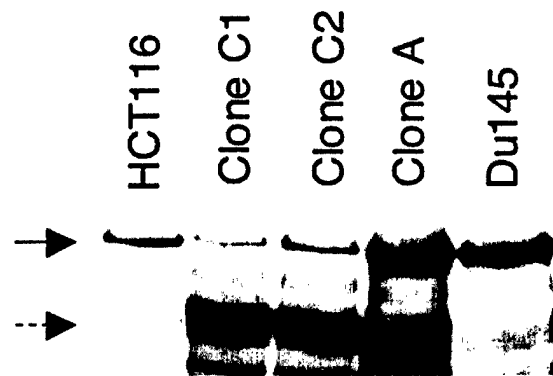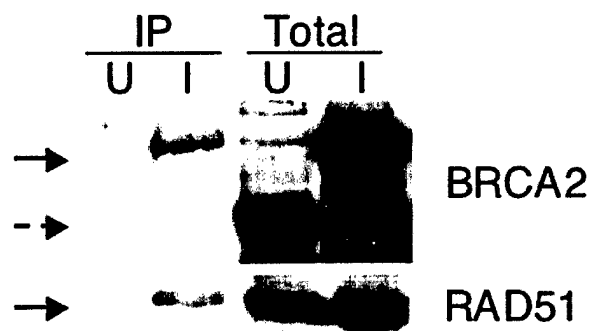
Figure 2   A

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7
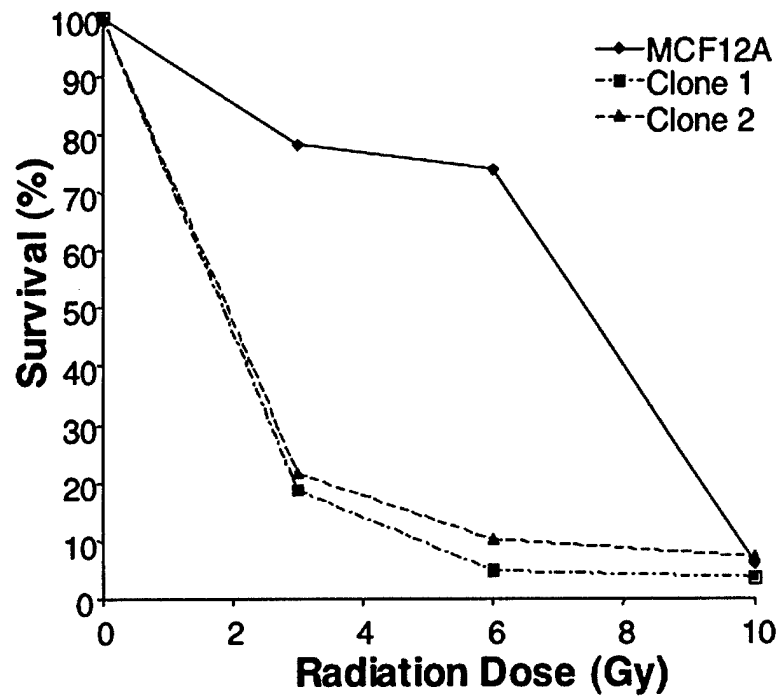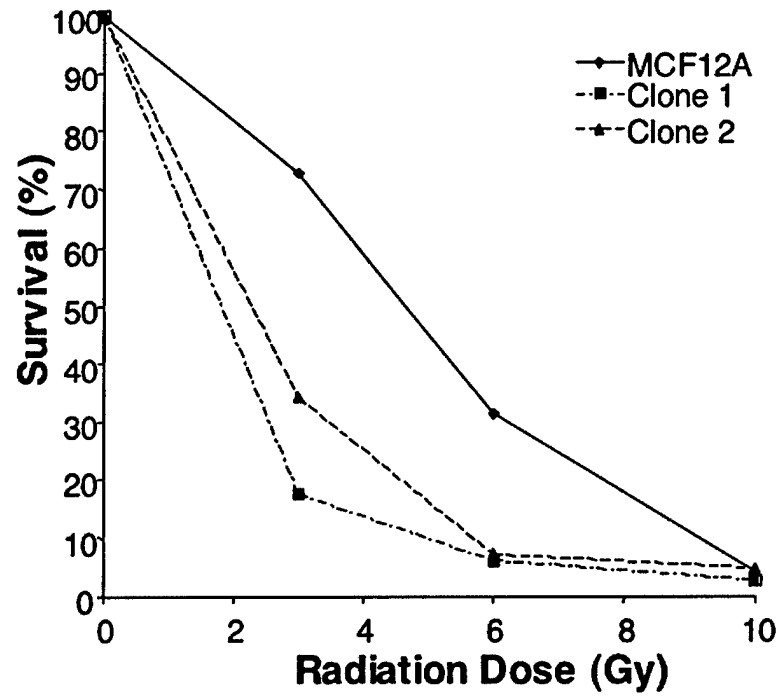
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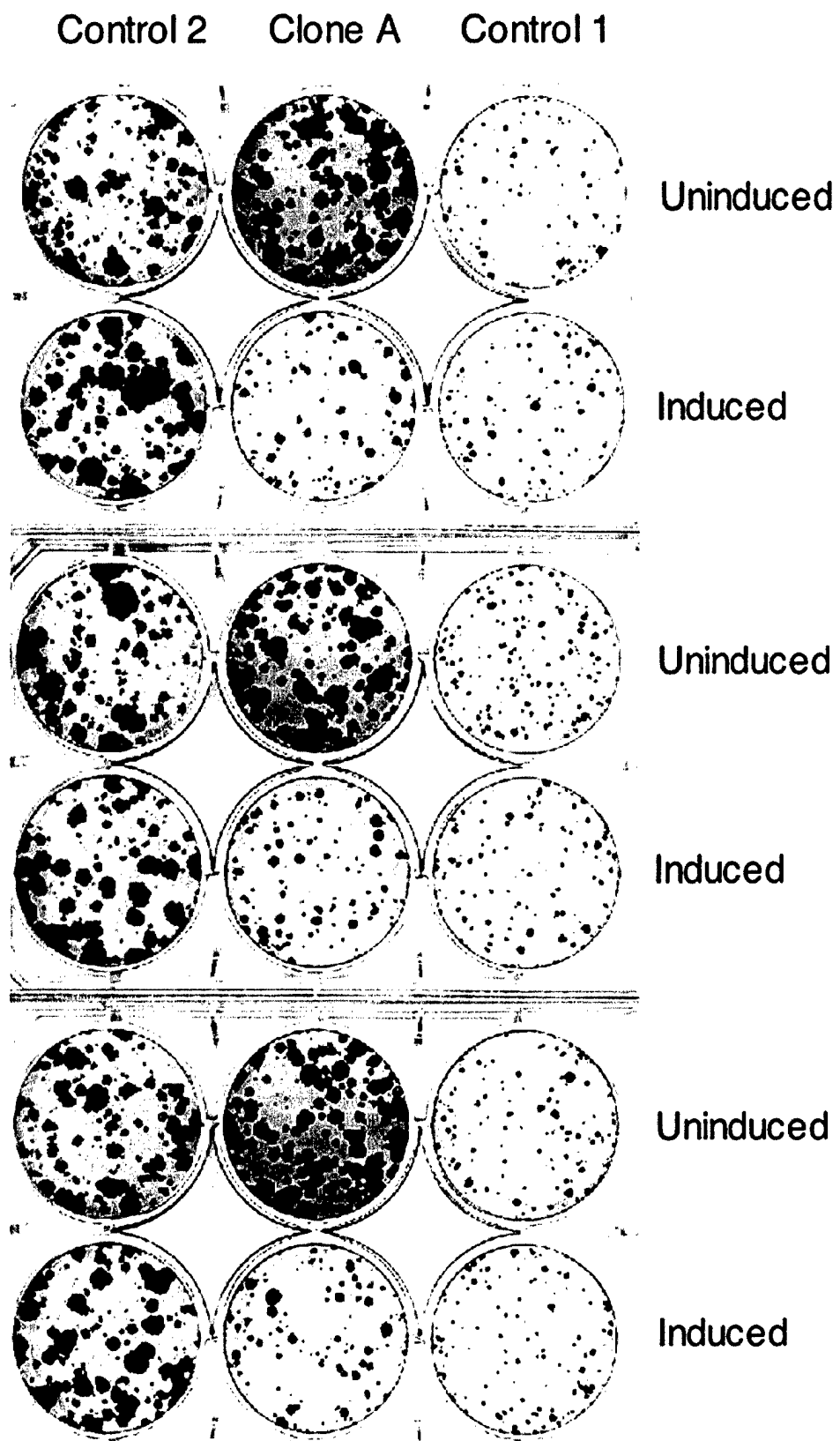
Figure 9

Figure 10

A

siRNA
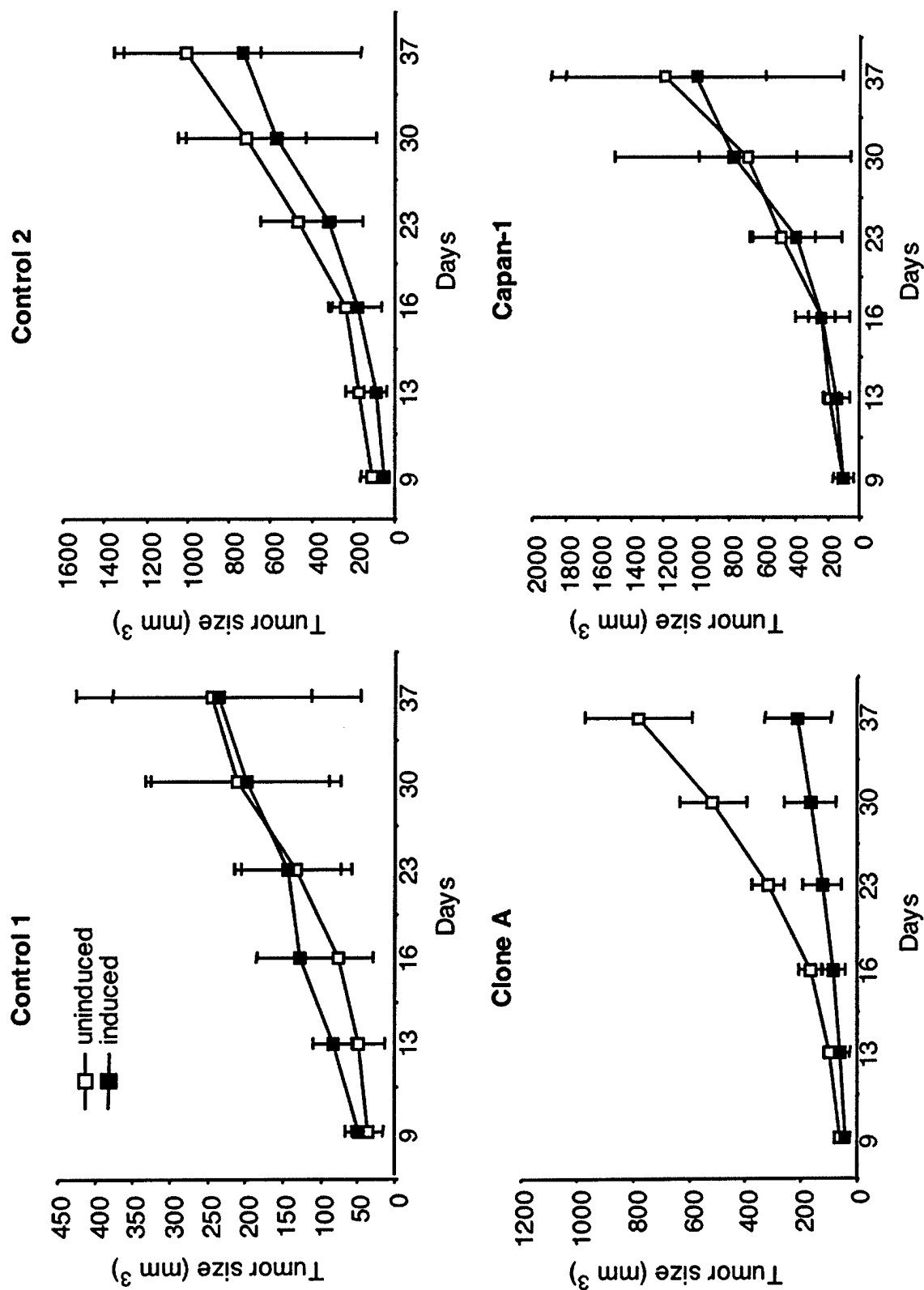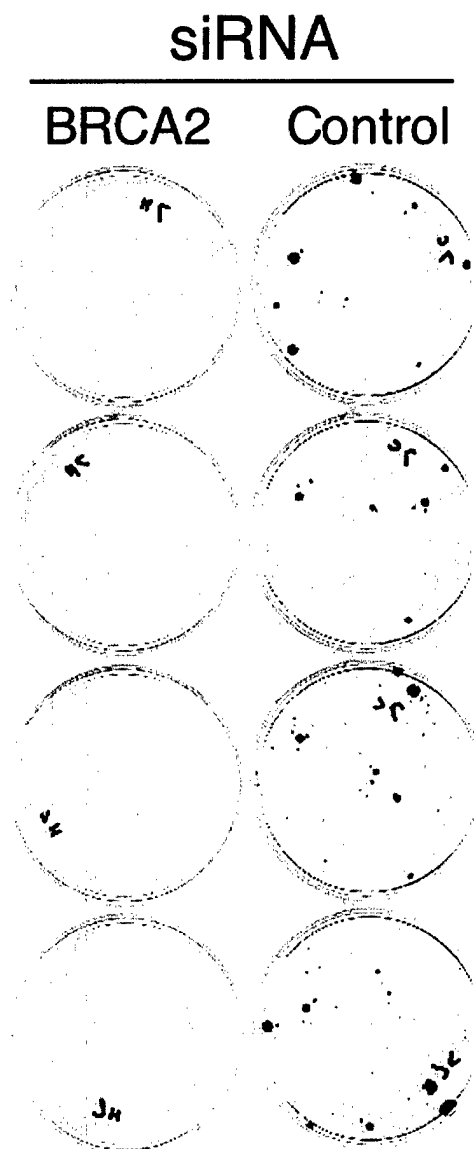
BRCA2	Control

B

Experiment	*BRCA2*	Control
1	0	21
2	0	6
3	0	5
4	0	21

Key Research Accomplishments

- We have established Capan-1 derivatives that express wild-type BRCA2 constitutively or under the regulation of tetracycline.

- We show that expression of wild-type BRCA2 does not reduce the sensitivity of Capan-1 cells to DNA damaging agents.

- We show that expression of wild-type BRCA2 reduces the growth of Capan-1 cells in vitro and in vivo.

- We have established MCF12A derivatives that express reduced levels of BRCA2.

- We show that reduction of BRCA2 levels results in MCF12A cells to be more sensitive to γ-irradiation.

- Our results suggest that BRCA2 may be essential of the growth of MCF7 cells.

Reportable Outcomes

- Results of characterization of wild-type BRCA2-expressing Capan-1 derivatives have been reported in the 51st Annual Meeting of the American Society of Human Genetics and the 3rd Era of Hope Meeting.

- A paper showing the effect of expressing wild-type BRCA2 on the growth of Capan-1 cells has been published (Cancer Res., 62: 1311-1314, 2002).

Conclusions

We have accomplished all tasks except the establishment and characterization of MCF7 derivatives that express reduced levels of BRCA2 this is because that BRCA2 appears to be essential for the growth of MCF7 cells.

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Appendix:

None.